Muteins of the bilin-binding protein

The present invention relates to muteins of the bilin-binding protein which are capable of binding digoxigenin and to fusion proteins of such muteins, to methods for preparing muteins of this kind and their fusion proteins and also to the use thereof for detecting or binding biomolecules labeled with digoxigenin.

In molecular biology, the digoxigenin group is these days a very common instrument for nonradioactive detection of nucleic acids, proteins and other biomolecules. For this purpose, the biomolecule is, mostly covalently, modified with a reactive digoxigenin derivative, thus allowing subsequent detection of the molecule using an antibody directed against the digoxigenin group or a conjugate of an appropriate antibody fragment and a reporter enzyme, according to generally used methods in biochemistry.

The skilled worker knows quite a number of reactive digoxigenin derivatives, which are in part also commercially available. For example, digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid N-hydroxysuccinimide ester (DIG-NHS), digoxigenin-3-O-succinyl-ε-amino-caproic acid N-hydroxysuccinimide ester and 3-amino-3-deoxydigoxigenin-hemisuccinimide succinimidyl ester are suitable for covalent coupling to proteins, in particular to the amino groups of exposed lysine side chains. Using 3-iodoacetylamino-3-deoxydigoxigenin it is possible to label especially thiol groups in proteins or in other biomolecules in a selective manner with the digoxigenin group. It is possible to couple synthetic oligodeoxynucleotides to the same reactive digoxigenin derivatives, as long as they have been equipped with suitable free amino or thiol groups during synthesis.

In addition, cis-platinum complexes of digoxigenin derivatives (DIG Chem-Link reagent) or digoxigenin derivatives containing carbodiimide groups (disclosed in the European patent specification EP 0 806 431 A2) are suitable for the direct labeling of nucleic acids. Alternatively, it is possible in the case of deoxyribonucleic acids to label said deoxyribonucleic acids during a template-dependent enzymatic synthesis with the aid of a DNA polymerase and a deoxynucleotide triphosphate coupled to the digoxigenin group, for example digoxigenin-11-dUTP, digoxigenin-11-ddUTP or digoxigenin-16-dATP. Analogously, digoxigenin-11-UTP is suitable for incorporation into enzymatically synthesized RNA. Moreover, it is possible to label oligodeoxynucleotides with the digoxigenin group directly in the automated DNA synthesis by using suitable activated building blocks, for example so-called "virtual nucleotides". Digoxigenin group-coupled nucleic acids of this kind are suitable as nonradioactive gene probes for detection of complementary nucleotide sequences by hybridization, for example in Northern or Southern blots (disclosed in the European patent specification EP 0 324 474 A1).

Digoxigenin group-labeled proteins or glycoproteins are particularly useful for determining, for example, relevant antigens or antibodies directed there against in immunochemical assay methods such as ELISA (enzyme-linked immunosorbent assay). The biomolecule conjugated with the digoxigenin group is actually detected using an anti-digoxigenin antibody, normally in the form of a conjugate of the Fab fragment of said antibody with a suitable enzyme, such as, for example, alkaline phosphatase or horseradish peroxidase, as label. The enzymatic activity then serves for quantification via catalysis of a chromogenic, fluorogenic or chemiluminescent reaction. Various antibodies against the digoxigenin group are known (Mudgett-Hunter et al.,

J. Immunol. 129 (1982), 1165-1172; Jeffrey et al., J. Mol. Biol. 248 (1995), 344-360).

The use of antibodies, however, has several disadvantages. Hence, the preparation of monoclonal antibodies in hybridoma cell cultures is complicated, and the proteolysis for the Fab fragment and also the production of conjugates with reporter enzymes requires additional difficult processing steps. But even the production of antibodies by genetic engineering is not simple, and the main reason for this is that antibodies as well as their antigen-binding fragments are composed of two different polypeptide chains in a structurally complicated manner. For the genetic manipulation of antibodies it is therefore necessary to handle two genes simultaneously. Moreover, the yield of correctly folded antibody fragments produced by genetic engineering is often low. As is known to the skilled worker, this is even more so when recombinant fusion proteins are to be prepared from Fab fragments of antibodies and enzymes.

It was therefore the object of the invention to develop alternative polypeptide reagents for detection of the digoxigenin group, which can be produced in a simple manner.

In an evolutionary research approach, it has surprisingly been found now that muteins of the bilin-binding protein, which is structurally based on a single polypeptide chain (Schmidt and Skerra, Eur. J. Biochem. 219 (1994), 855-863), are suitable for detecting the digoxigenin group by binding with high affinity, whereby the recognition of digoxigenin is astoundingly selective compared with other steroids.

The present invention thus relates to a polypeptide, selected from muteins of the bilin-binding protein, which is characterized in that it

(a) is able to bind digoxigenin or digoxigenin conjugates,

(b) does not bind ouabain, testosterone, and 4-aminofluorescein and

(c) has an amino acid substitution at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 of the bilin-binding protein.

In this connection, preference is given to digoxigenin-binding muteins which have an amino acid substitution at at least 4 to 7 or, preferably, at least 8 to 12 of the sequence positions defined above. A particularly preferred mutein is the polypeptide which has the amino acid sequence depicted as SEQ ID NO. 15.

Outside the region of the amino acid positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 the muteins of the present invention may correspond to the amino acid sequence of the bilin-binding protein from *Pieris brassicae*. On the other hand, the amino acid sequence of the polypeptides of the invention may also have differences to the bilin-binding protein apart from said positions. Bilin-binding protein sequence variants of this kind comprise naturally occurring and also artificially generated variants, and the deviations mean substitutions, insertions, deletions of amino acid residues and also N- and/or C-terminal additions.

For example, the inventive muteins of the bilin-binding protein may have amino acid substitutions which prevent oligomerization of the bilin-binding protein, such as the Asn(1)->Asp substitution, or suppress proteolytic cleavage within the polypeptide chain, which may occur during production in *E. coli*, such as, for example, by the Lys(87)->Ser substitution. Furthermore, the mutations Asn(21)->Gln and Lys(135)->Met may be introduced into the nucleic acid coding for the muteins

of the bilin-binding protein, in order to facilitate, for example, cloning of a gene segment via two new *Bst*XI restriction cleavage sites at these positions. Likewise, the present invention relates to the specific introduction of amino acid substitutions within or outside the said positions, in order to generally improve particular properties of the mutein of the invention, for example its folding stability or folding efficiency or its resistance to proteases.

The ability of the polypeptides of the invention to bind digoxigenin or digoxigenin conjugates can be determined by common methods, for example ELISA, fluorescence titration, titration calorimetry, surface plasmon resonance measurements or blotting methods, for example Western blotting, Southern blotting or Northern blotting. Blotting methods may be used in order to transfer conjugates of digoxigenin with proteins or nucleic acids to a membrane and then detect said conjugates using one of the muteins of the invention, a conjugate of this mutein or a fusion protein of this mutein.

A quantitative parameter for binding affinity is provided by established thermodynamic parameters such as, for example, the affinity constant or dissociation constant for the complex of mutein and bound ligand, for example digoxigenin. However, it is also possible to determine the binding ability qualitatively, for example based on the intensity of a binding signal due to a chromogenic reaction or of a colored precipitate which is obtained with the aid of one of said blotting methods.

Preferred muteins of the invention are obtainable in a two-stage evolutionary process. Random mutagenesis of the bilin-binding protein at at least one, preferably at at least 4 to 7, and particularly preferably at at least 8 to 12, of the sequence positions 28, 31, 34,

35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 and subsequent simple or, preferably, repeated selection of muteins with affinity for the digoxigenin group from this library, preferably using free digoxigenin or digitoxigenin for competitive enrichment, provides muteins of the bilin-binding protein which recognize the digoxigenin group, but the affinity is still comparatively low. Renewed mutagenesis of such a mutein at at least one, preferably at at least 3 or 4, or at all of amino acid positions 28, 31, 34, 35, 36 and 37, now followed by a simple or, preferably, repeated enrichment by formation of a complex with the digoxigenin group and by subsequent dissociation of the formed complex in an acidic or basic milieu, then results in obtaining muteins having substantially higher affinity for the digoxigenin group. The digoxigenin group is preferably present as a digoxigenin/biotin double conjugate during said enrichment.

Surprisingly, it has now been found that the affinity constant between such polypeptides of the invention and digoxigenin is at least 10^7 M^{-1}. This means in other words that the dissociation constant of the complex between the polypeptide of the invention and digoxigenin is 100 nM or less. Individual species even show dissociation constants of 35 nM or less, as illustrated in the Examples.

Besides digoxigenin, the inventive muteins of the bilin-binding protein can also bind digoxigenin derivatives as ligands, for example digoxin, digitoxin or digitoxigenin. Furthermore, the inventive muteins of the bilin-binding protein may bind conjugates of said chemical compounds, i.e. nucleic acids, polypeptides, carbohydrates, other natural or synthetic biomolecules, macromolecules or low molecular weight compounds which are covalently linked or linked via a metal complex to digoxigenin, digoxin, digitoxin or digitoxigenin.

Preference is given to using for the preparation of such conjugates the reactive derivatives of digoxigenin, digoxin, digitoxin or digitoxigenin, which are known to the skilled worker and are stated, for example, further above.

Preferred muteins of the invention, which were obtained by the two-stage process described, show, compared with the affinity for digoxigenin, an even higher affinity for digitoxin or digitoxigenin, whose steroid system differs from that of digoxigenin only by the absence of a hydroxyl group. Surprisingly, these muteins show distinctive specificity with respect to the digoxigenin or digitoxigenin group, and this is shown by the fact that other steroids or steroid groups such as ouabain or testosterone are bound with much less affinity, if at all. Fluorescein derivatives such as 4-amino-fluorescein, too, are evidently not bound. This means that ouabain, testosterone or 4-aminofluorescein in each case exhibit a dissociation constant of at least 10 µM, preferably at least 100 µM, with respect to the inventive muteins of the bilin-binding protein.

This property of specificity distinguishes said muteins considerably from other muteins of the bilin-binding protein and also from antibodies directed against the digoxigenin group, such as, for example, the antibody 26-10 (Chen et al., Protein Eng. 12 (1999), 349-356), which binds ouabain with substantial affinity, and gives the inventive muteins of the bilin-binding protein a particular advantage. It is surprising that particularly the additional amino acid substitutions at positions 28, 31, 34, 35, 36, and 37 lead to the preferred muteins of the bilin-binding protein. Preference is therefore given to those muteins which carry at least one, preferably at least 3 or 4, or all of the amino acid substitutions Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile and Glu(37)->Thr.

Particularly preferred muteins of the invention carry, when compared to the bilin-binding protein, at least one, at least 4 to 7, or, preferably, at least 8 to 12 of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln, Asn(97)->Gly, Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met and Phe(127)->Leu. The representation chosen indicates in each case first the amino acid in the natural bilin-binding protein (SWISS-PROT database accession code P09464) together with the sequence position for the mature polypeptide in brackets, and the corresponding amino acid in a mutein of the invention is stated after the arrow. Very particularly preferred muteins according to this invention carry all of the amino acid substitutions mentioned.

Surprisingly, position 93 of the bilin-binding protein is unchanged in the muteins of the invention, although this amino acid, too, had been affected by the mutagenesis for preparing the random library. Preferred muteins of the bilin-binding protein therefore carry the amino acid Val at said position.

It is an advantage for particular detection methods to use the muteins of the bilin-binding protein of the present invention in a labeled form. Accordingly, this invention further relates to a polypeptide of the invention which is characterized in that it carries at least one label. Suitable labeling groups are known to the skilled worker and include enzyme label, radioactive label, fluorescent label, chromophoric label, (bio)luminescent label or a label containing haptens, biotin, metal complexes, metals or colloidal gold. Very generally, labeling is possible with substances or enzymes which generate a determinable substance in a chemical or enzymatic reaction. In this

connection it is possible to couple all known labels for antibodies to the muteins of the invention, too.

A possibility which is particularly advantageous for practical application is to use the inventive muteins of the bilin-binding protein in the form of fusion proteins. Techniques for preparing such fusion proteins by means of genetic engineering methods are known to the skilled worker. Suitable fusion partners for the muteins of the invention would be enzymes and other polypeptides, proteins or protein domains. Such fusions would be suitable for providing the mutein of the bilin-binding protein with additional properties such as, for example, enzymatic activity or affinity for other molecules, such as proteins, macromolecules or low molecular weight ligands.

For example, fusions are possible with enzymes which catalyze chromogenic or fluorogenic reactions or may serve for the release of cytotoxic agents. Further examples for fusion partners which may be advantageous in practice are binding domains such as the albumin-binding domain or the immunoglobulin-binding domain of protein G or protein A, antibody fragments, oligomerization domains, toxins or other binding proteins and functional parts thereof and also affinity peptides such as, for example, the Strep-tag or the Strep-tag II (Schmidt et al., J. Mol. Biol. 255 (1996), 753-766). Suitable fusion partners are also proteins having particular chromogenic or fluorogenic properties, such as, for example, green fluorescent protein. Another suitable fusion partner would be the coat protein III of a filamentous bacteriophage, such as M13, f1 or fd, or a fragment of said coat protein.

Very generally, the term fusion protein is intended here to mean also those inventive muteins of the bilin-binding protein, which are equipped with a signal sequence. Signal sequences at the N-terminus of the

polypeptide of the invention may serve for the purpose of directing said polypeptide during biosynthesis into a particular cell compartment, for example the *E. coli* periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell, or into the medium surrounding the cell. The signal sequence is typically cleaved off by a signal peptidase. In addition, it is also possible to use other signal or targeting sequences which need not necessarily be located at the N-terminus of the polypeptide and which make it possible to locate said polypeptide in specific cell compartments. A preferred signal sequence for secretion into the *E. coli* periplasm is the OmpA signal sequence. A large number of further signal sequences and also targeting sequences are known in the prior art.

An advantage of the inventive muteins of the bilin-binding protein is the suitability of both their N-terminus and their C-terminus for preparing fusion proteins. In contrast to antibodies, in which the N-terminus of both the light and the heavy immunoglobulin chain are in spatial proximity to the antigen binding site, it is possible to use in the polypeptides of the invention both ends of the polypeptide chain for the preparation of fusion proteins, without adversely affecting ligand binding.

The invention therefore also relates to fusion proteins of muteins of the bilin-binding protein in which an enzyme, another protein or a protein domain, a signal sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner. The invention yet further relates to fusion proteins of bilin-binding protein muteins or of fusion proteins with the amino terminus of bilin-binding protein muteins in which an enzyme, another protein or a protein domain, a targeting sequence and/or an affinity peptide is fused to the carboxy terminus of the polypeptide in an operable manner.

A preferred enzyme for constructing the fusion proteins of the invention is bacterial alkaline phosphatase (Sowadski et al., J. Mol. Biol. 186 (1985) 417-433), which may be attached either at the N-terminus or at the C-terminus of a mutein of the bilin-binding protein. In addition, such a fusion protein may carry a signal sequence such as, for example, OmpA or PhoA, which effects secretion of said fusion protein into the *E. coli* periplasm, where the disulfide bonds of the polypeptide chain may form efficiently. Furthermore, it may be equipped with an affinity peptide such as, for example, the Strep-tag II, which allows easy purification of said fusion protein. Specific fusion proteins of the invention are described in the Examples. An advantage of a fusion protein of this kind is its ability to catalyze directly a chromogenic, fluorogenic or chemiluminescent detection reaction, which simplifies its use for detection of the digoxigenin group.

Another advantage of using alkaline phosphatase for constructing fusion proteins of the invention is the fact that this enzyme forms a stable homodimer and, consequently, confers the property of bivalence on the bilin-binding protein mutein as part of the fusion protein. In this way, binding of the digoxigenin group may result in an avidity effect, which increases detection sensitivity. Such an avidity effect can be expected in particular if the digoxigenin-labeled molecule is adsorbed to a solid phase, is present in oligomeric or membrane-bound form or is conjugated with a plurality of digoxigenin groups. Analogously, other homodimeric enzymes are suitable for preparing bivalent fusion proteins containing the inventive muteins of the bilin-binding protein.

Apart from bacterial alkaline phosphatase, it is also possible to use phosphatases from eukaryotic organisms,

such as, for example, calf intestine phosphatase (CIP), for preparing fusion proteins of the invention. Said phosphatases are frequently distinguished by higher enzymatic activity (Murphy and Kantrowitz, Mol. Microbiol. 12 (1994), 351-357), which may result in higher detection sensitivity. It is also possible to use mutants of bacterial alkaline phosphatase, which have improved catalytic activity (Mandecki et al., Protein Eng. 4 (1991), 801-804), for constructing fusion proteins of the invention. Other enzymes known to the skilled worker which catalyze chromogenic, fluorogenic or chemiluminescent reactions, such as, for example, β-galactosidase or horseradish peroxidase, are also suitable for preparing fusion proteins of the invention. Moreover, all these enzymes may likewise be employed for labeling muteins of the bilin-binding protein by conjugating them, for example by using common coupling reagents, with the separately obtained mutein or a fusion protein of the mutein.

In another aspect, the present invention relates to a nucleic acid which comprises a sequence coding for a mutein or a fusion protein of a mutein of the bilin-binding protein. This nucleic acid may be part of a vector on which an operatively functional environment for expressing the nucleic acid is present. A large number of suitable vectors is known from the prior art and is not described in detail here. An operatively functional environment means those elements which allow, assist, facilitate and/or increase transcription and/or subsequent processing of an mRNA. Examples of elements of this kind include promoters, enhancers, transcription initiation sites, and transcription termination sites, translation initiation sites, polyadenylation signals, etc. In a preferred embodiment, such nucleic acids of the invention comprise a nucleic acid sequence which encodes the polypeptide sequence depicted as SEQ ID NO:15. Owing to the degeneracy of the genetic code, it is clear to the

skilled worker that the nucleotide sequence stated as SEQ ID NO:15 represents only a single nucleotide sequence from the group of nucleotide sequences encoding the polypeptide according to SEQ ID NO:15.

The nucleic acid of the invention or its environment may be designed such that biosynthesis of the polypeptide takes place in the cytosol, in which case the polypeptide sequence being preceded, where appropriate, by a start methionine. In a preferred embodiment, however, an N-terminal signal sequence is used, in particular the OmpA or PhoA signal sequence, in order to direct the polypeptide of the invention into the *E. coli* periplasm, where the signal sequence is cleaved off by the signal peptidase and the polypeptide chain is able to fold with oxidative formation of the disulfide bonds. Eukaryotic signal sequences may be used in order to secrete the polypeptide of the invention in a eukaryotic host organism.

In principle, both prokaryotic, preferably *E. coli*, and eukaryotic cells such as, for example, yeasts are considered for expression of the nucleic acid of the invention.

In yet another aspect, the present invention relates to a method for preparing an inventive mutein or fusion protein of a mutein of the bilin-binding protein, which method is characterized in that the nucleic acid coding for the mutein or the fusion protein of a mutein of the bilin-binding protein is expressed in a bacterial or eukaryotic host cell and the polypeptide is obtained from the cell or the culture supernatant. For this purpose, normally a suitable host cell is first transformed with a vector which comprises a nucleic acid coding for a polypeptide of the invention. The host cell is then cultured under conditions under which

bio-synthesis of the polypeptide occurs, and the polypeptide of the invention is obtained.

With respect to the preparation method, it must be taken into account that the inventive muteins of the bilin-binding protein have two structural disulfide bonds and that additional disulfide bonds may be present in corresponding fusion proteins. The formation of said disulfide bonds, which takes place during protein folding, is normally ensured if the polypeptide of the invention is directed with the aid of a suitable signal sequence into a cell compartment containing an oxidizing thiol/disulfide redox milieu, for example into the bacterial periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell. In this respect, the polypeptide of the invention can be liberated by cell fractionation or obtained from the culture supernatant. It is possible, where appropriate, to increase the folding efficiency by overproducing protein disulfide isomerases, for example *E. coli* DsbC protein, or proteins that assist folding.

On the other hand, it is possible to produce a polypeptide of the invention in the cytosol of a host cell, preferably *E. coli*. The said polypeptide may then be obtained, for example, in the form of inclusion bodies and afterwards be renatured *in vitro*. Depending on the intended use, the protein can be purified by means of various methods known to the skilled worker. A suitable method for purifying the inventive muteins of the bilin-binding protein is, for example, affinity chromatography using a column material which carries digoxigenin groups. In order to purify fusion proteins of the muteins of the bilin-binding protein, it is possible to utilize the affinity properties of the fusion protein, which are known from the prior art, for example those of the Strep-tag or the Strep-tag II (Schmidt and Skerra, J. Chromatogr. A 676 (1994), 337-345; Voss and Skerra, Protein Eng. 10 (1997), 975-982),

those of the albumin binding domain (Nygren et al., J. Mol. Recogn. 1 (1988), 69-74) or those of alkaline phosphatase (McCafferty et al., Protein Eng. 4 (1991) 955-961). The fact that the muteins of the bilin-binding protein consist only of a single polypeptide chain is advantageous for the methods for preparing the polypeptides of the invention, since no care needs to be taken either of synthesizing several different polypeptide chains within a cell simultaneously or of different polypeptide chains associating with one another in a functional manner.

The possibilities for practical application of the inventive muteins of the bilin-binding protein essentially correspond to those for conventional antibodies or antibody fragments with binding affinity for digoxigenin. Accordingly, the invention also relates to the use of a mutein of the invention or of a fusion protein of a mutein of the bilin-binding protein in a method for detecting, determining, immobilizing or removing digoxigenin or conjugates of digoxigenin with proteins, nucleic acids, carbohydrates, other biological or synthetic macromolecules or low molecular weight chemical compounds.

The inventive muteins of the bilin-binding protein or their fusion proteins can be used in detection methods essentially in a manner analogous to corresponding detection methods known for anti-digoxigenin antibodies and also fragments and/or conjugates thereof. In a further aspect, the present invention therefore relates to a method for detecting the digoxigenin group, in which method a mutein of the bilin-binding protein or a fusion protein of a mutein of the bilin-binding protein is brought into contact with digoxigenin or with digoxigenin conjugates under conditions suitable for effecting binding of the mutein to the digoxigenin group and the mutein or the fusion protein of the mutein is determined.

For this purpose, the mutein may be labeled directly, for example by covalent coupling. It is, however, also possible to use indirect labeling, for example by means of labeled antibodies against the bilin-binding protein or muteins thereof or against domains of fusion proteins of these muteins. The use of inventive fusion proteins containing an enzyme, for example alkaline phosphatase, instead of a labeled mutein of the bilin-binding protein is particularly advantageous. In this case, it is possible to design the determination method with a particularly small number of process steps, whereby, for example, the ability of the enzyme as part of the fusion protein to catalyze a chromogenic, fluorogenic or luminescent detection reaction may be directly utilized. Here, the fact that such fusion proteins are readily available is a particular advantage compared with corresponding fusion proteins of conventional antibodies. Utilization of the above-described avidity effect in the case of an oligomeric fusion protein is a further advantage in such a method.

It is possible to carry out a method for determining the digoxigenin group, for example, for qualitatively detecting nucleic acids conjugated with the digoxigenin group in Southern or Northern blots or proteins conjugated with the digoxigenin group in Western blots. A determination method may also be carried out quantitatively for detecting proteins conjugated with the digoxigenin group in an ELISA. In addition, a determination method of the invention is also suitable for indirect detection of proteins not conjugated with digoxigenin or of other molecules by using a binding protein which is directed against the protein or molecule, for example an antibody or its fragment, and which is conjugated with the digoxigenin group. Indirect detection of the nucleic acids not conjugated with digoxigenin is also possible by using a gene probe which hybridizes with said nucleic acid and which is

conjugated with the digoxigenin group. An application in medical diagnostics or therapy results furthermore from the determination of digoxigenin, digoxin, digitoxin or digitoxigenin, without these ligands having to be conjugated with another molecule.

The muteins of the invention or fusion proteins thereof may also be used for immobilizing a molecule conjugated with the digoxigenin group. This immobilization is preferably carried out on solid phases coated with the muteins or their fusion proteins, such as, for example, microtiter plates, immunosticks, microbeads made of organic, inorganic or paramagnetic materials, or sensor surfaces.

Correspondingly, it is likewise possible to use the muteins of the invention or fusion proteins thereof for removing digoxigenin, digoxin, digitoxin or digitoxigenin, or a molecule conjugated with one of these compounds. In this case, in addition to the solid phases mentioned, column materials are also considered for coating with the muteins or their fusion proteins. Preferably, said coating is carried out on suitable column materials by coupling by means of chemically reactive groups. Column materials coated in this way may be used for removing from a solution substances conjugated with digoxigenin groups and also, where appropriate, complexes of such substances with other molecules.

Thus, it is possible, for example, to remove antigens from a solution by adding to the solution antibodies which are directed against the antigens and are conjugated with the digoxigenin group, and contacting the resulting solution with said column material under conditions under which complex formation between the digoxigenin groups and an inventive mutein of the bilin-binding protein or its fusion protein occurs. Following such a removal, it is also possible, where

appropriate, to elute the substance conjugated with the digoxigenin. This elution may be carried out by competition with digoxin, digoxigenin, digitoxin or digitoxigenin and also, for example, by lowering or increasing the pH of the solution. In a competitive elution it is possible to utilize in an advantageous manner the higher binding affinity of the muteins of the invention for digitoxigenin or digitoxin compared with the digoxigenin group. In this way it is possible to isolate or purify a substance conjugated with digoxigenin.

The invention is further illustrated by the following Examples and attached drawings, in which:

Figure 1 represents in each case a fluorescence titration of the Strep-tag II-fused mutein DigA16 with the ligands digoxigenin, digitoxigenin, and ouabain;

Figure 2 depicts diagrammatically the expression vectors pBBP27 (A) and pBBP29 (B) for preparing fusion proteins of mutein DigA16 with alkaline phosphatase;

Figure 3 demonstrates quantitative detection of biomolecules conjugated with digoxigenin groups by fusion proteins of mutein DigA16 with alkaline phosphatase in an ELISA;

Figure 4 shows qualitative detection of biomolecules conjugated with digoxigenin groups by fusion proteins of mutein DigA16 with alkaline phosphatase on a Western blot.

Figure 1 shows the graphic representation of results from Example 3 in which different concentrations of the steroids digoxigenin (squares), digitoxigenin (circles) and ouabain (rhomboids) were added to a 1 µM solution

of mutein DigA16. The particular protein fluorescence intensities were measured at an excitation wavelength of 295 nm and an emission wavelength of 345 nm and plotted as a function of the actual total steroid concentration in the particular reaction mixture. Finally, the data points were fitted to a regression curve by means of non-linear regression.

Figure 2 shows a drawing of the expression vectors pBBP27 (A) and pBBP29 (B). pBBP27 codes for a fusion protein of bacterial alkaline phosphatase with its own signal sequence, a peptide linker having the sequence Pro-Pro-Ser-Ala, the mutein DigA16 and also the Strep-tag II affinity tag. The corresponding structural gene is followed by the *dsbC* structural gene (including its ribosomal binding site) from *E. coli* (Zapun et al., Biochemistry 34 (1995), 5075-5089) as second cistron. The artificial operon formed in this way is under joint transcriptional control of the tetracycline promoter/operator ($tet^{p/o}$) and ends at the lipoprotein transcription terminator (t_{1pp}). Further vector elements are the origin of replication (ori), the intergenic region of filamentous bacteriophage f1 (f1-IG), the ampicillin resistance gene (bla) coding for β-lactamase and the tetracycline repressor gene (tetR). pBBP29 codes for a fusion protein of the OmpA signal sequence, the mutein DigA16, the Strep-tag II affinity tag, a peptide linker consisting of five glycine residues, and bacterial alkaline phosphatase without its N-terminal amino acid arginine. The vector elements outside this region are identical to vector pBBP27.

Figure 3 shows a graphic representation of the data from Example 4 in which digoxigenin groups were detected quantitatively with the aid of mutein DigA16 fusion proteins as gene products of vectors pBBP27 (closed symbols) and pBBP29 (open symbols). Here, the digoxigenin groups were coupled on the one hand to bovine serum albumin (BSA, squares) or, on the other

hand, to chicken egg albumin (ovalbumin, triangles). The control data shown are those obtained when using underivatized bovine serum albumin and the fusion protein encoded by pBBP27 (open circles). The enzymatic activity corresponding to the particular bound fusion protein was monitored spectrophotometrically at 405 nm on the basis of p-nitrophenyl phosphate hydrolysis. Curve fitting was carried out by non-linear regression with the aid of the Kaleidagraph computer program (Abelbeck Software) by means of the equation

$$[P \cdot L] = [L]_t [P]_t / (K_d + [P]_t).$$

Here, $[P]_t$ corresponds to the total fusion protein concentration used in the particular microtiter plate well. [P•L] is determined on the basis of the enzymatic activity of alkaline phosphatase. The total concentration of digoxigenin groups $[L]_t$, constant within a concentration series, per well and the dissociation constant K_d were fitted as parameters by non-linear regression.

Figure 4 shows the result of a Western blot experiment from Example 4 for qualitative detection of biomolecules conjugated with digoxigenin groups by means of the mutein DigA16 fusion proteins encoded by pBBP27 (lanes 1 and 2) and pBBP29 (lanes 3 and 4). For comparison, a 15% strength SDS polyacrylamide gel of the biomolecules, stained with Coomassie Brilliant Blue, is also shown (lanes 5 and 6). Here, a mixture of 0.5 µg of underivatized BSA, underivatized ovalbumin and underivatized RNaseA was fractionated in each case in lanes 1, 3 and 5. A mixture of 0.5 µg of BSA coupled to digoxigenin groups, ovalbumin coupled to digoxigenin groups and RNaseA coupled to digoxigenin groups was fractionated in each case in lanes 2, 4 and 6.

Examples

Unless stated otherwise, the genetic engineering methods familiar to the skilled worker, as described, for example, in Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989), Cold Spring Harbor Press) were used.

Example 1: Preparation of a library for muteins of the bilin-binding protein, phagemid presentation and selection of a mutein with binding affinity for digoxigenin

A library for muteins of the bilin-binding protein was prepared by subjecting the amino acid sequence positions 34, 35, 36, 37, 58, 60, 69, 88, 90, 93, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein to a concerted mutagenesis in multiple steps by means of the polymerase chain reaction (PCR). The PCR reactions were initially carried out in two separate amplification steps in a volume of in each case 50 µl, and 10 ng of pBBP20 phasmid DNA (SEQ ID NO:1) as template using in each case 25 pmol of two primers (SEQ ID NO:2 and SEQ ID NO:3 in one mixture and SEQ ID NO:4 and SEQ ID NO:5 in a second mixture) which had been synthesized according to the generally known phosphoramidite method were used.

Furthermore, the reaction mixture contained 5 µl of 10xTaq buffer (100 mM Tris/HCl pH 9.0, 500 mM KCl, 1% v/v Triton X-100), 3 µl of 25 mM $MgCl_2$ and 4 µl of dNTP mix (2.5 mM dATP, dCTP, dGTP, dTTP). After filling up with water, the mixture was overlaid with mineral oil and heated to 94°C in a programmable thermostating block for 2 min. Then 2.5 u of *Taq* DNA polymerase (5 u/µl, Promega) were added and 20 temperature cycles of 1 min at 94°C, 1 min at 60°C and 1.5 min at 72°C were carried out, followed by an incubation at 60°C for 5 min. The desired amplification products were isolated via preparative agarose gel electrophoresis from low melting point agarose (Gibco BRL), using the Jetsorb

DNA extraction kit (Genomed) according to the manufacturer's instructions.

A relevant section of the pBBP20 nucleic acid sequence is shown together with the encoded amino acid sequence as SEQ ID NO:1 in the sequence listing. The section starts with a hexanucleotide sequence which was obtained by ligating an *Xba*I overhang with an *Spe*I overhang complementary thereto and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75 whose complete nucleotide sequence is stated in the German Offenlegungsschrift DE 44 17 598 A1.

The subsequent amplification step was carried out in a 100 µl mixture, and in each case approx. 6 ng of the two isolated fragments as template using 50 pmol of each of the two primers SEQ ID NO:6 and SEQ ID NO:7 and also 1 pmol of oligodeoxynucleotide SEQ ID NO:8. The remaining components of the PCR mixture were added in twice the amount, as in the preceding amplification steps. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min. The fragment obtained was again isolated by preparative agarose gel electrophoresis.

For cloning this fragment which represented the mutein library in the form of a mixture of nucleic acids it was first cut with the restriction enzyme *Bst*XI (New England Biolabs) according to the manufacturer's instructions. The nucleic acid fragment obtained (335 base pairs, bp) was purified again by means of preparative agarose gel electrophoresis. Analogously, pBBP20 vector DNA was cut with *Bst*XI and the larger of the two fragments (3971 bp) was isolated.

For ligation, 0.93 µg (4.2 pmol) of the PCR fragment and 11 µg (4.2 pmol) of the vector fragment were

incubated in the presence of 102 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 500 µl (50 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for two days. The DNA was then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for 3 days was followed by centrifugation (25 min, 16000 g, 4°C). The precipitate was washed in each case with 200 µl of ethanol (70% v/v, -20°C) and dried under vacuum. Finally, the DNA was taken up in 43.6 µl of TE/10 (1 mM Tris/HCl pH 8.0, 0.1 mM EDTA). The DNA concentration of the solution obtained was estimated by analytical agarose gel electrophoresis on the basis of the fluorescence intensity of the bands stained with ethidium bromide in comparison with a DNA size standard of known concentration.

Preparation of electrocompetent cells of the *E. coli* K12 strain XL1-Blue (Bullock et al., BioTechniques 5 (1987), 376-379) was carried out according to the methods described by Tung and Chow (Trends Genet. 11 (1995), 128-129) and by Hengen (Trends Biochem. Sci. 21 (1996), 75-76). 1 l of LB medium was adjusted to an optical density at 600 nm, OD_{600} = 0.08 by adding a stationary XL1-Blue overnight culture and incubated in a 3 l Erlenmeyer flask at 200 rpm and 26°C. After reaching OD_{600} = 0.6, the culture was cooled on ice for 30 min and then centrifuged at 4000 g and 4°C for 15 min. The cell sediment was washed twice with in each case 500 ml of ice cold 10% w/v glycerol and finally resuspended in 2 ml of ice cold GYT medium (10% w/v glycerol, 0.125% w/v yeast extract, 0.25% w/v tryptone).

Electroporation was carried out by using the Easyjec T Basic system (EquiBio) with the corresponding cuvettes (electrode distance 2 mm). All operational steps were

carried out in a cold room at 4°C. 5 to 6 µl of the above-described DNA solution (245 ng/µl) were in each case mixed with 40 µl of the cell suspension, incubated on ice for 1 min and then transferred into the cuvette. After electroporation, the suspension was immediately diluted in 2 ml of fresh ice-cold SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$) and agitated at 37°C and 200 rpm for 60 min. The cells were then sedimented at 3600 g for in each case 2 min, resuspended in 1 ml of LB medium containing 100 µg/ml of ampicillin (LB/Amp) and plated out at 200 µl each on agar plates (140 mm in diameter) with LB/Amp medium. Using a total of 10.7 µg of the ligated DNA in eight electroporation mixtures produced in this way $3.73 \cdot 10^8$ transformants which were distributed on 40 agar plates.

After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension. 50 ml of 2xYT/Amp medium prewarmed to 37°C were inoculated with 2.88 ml of said suspension so that the cell density was 1.0 OD_{550}. This culture was incubated at 37°C, 160 rpm for 6 h to reach a stationary cell density, and phasmid DNA was isolated with the aid of the plasmid Midi kit (Qiagen) according to the manufacturer's instructions. Finally, the DNA was taken up in 100 µl of TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and stored at 4°C for further use.

In order to prepare a library of recombinant phagemids (Kay et al., Phage Display of Peptides and Proteins - A Laboratory Manual (1996), Academic Press) which carry the muteins of the bilin-binding protein as a fusion with the truncated coat protein pIII, the phasmid DNA obtained in this way was used for transformation of electrocompetent cells of *E. coli* XL1-Blue.

Electroporation was carried out as described above with the aid of the Easyjec T Basic system. In a total of 13 mixtures, 40 µl of the cell suspension of electrocompetent cells were in each case transformed with in each case 2 µg of the DNA in a volume of 5 µl. After electroporation, the cell suspension obtained from each mixture was diluted immediately in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

The mixtures were combined (volume = 26 ml) and 74 ml of 2xYT medium and 100 µl of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added. The total number of transformants obtained was estimated at $1.1 \cdot 10^{10}$ by plating out 100 µl of a $1:10^5$ dilution of the obtained suspension on agar plates containing LB/Amp medium. After incubation at 37°C and 160 rpm for 60 min, the culture was infected with 500 µl of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene) and agitated at 37°C, 160 rpm for a further 60 min. Subsequently, 200 µl of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C and, after 10 min, anhydrotetracycline (50 µl of a 50 µg/ml stock solution in dimethylformamide, final concentration 25 µg/l) was added to induce gene expression. Finally, for production of the phagemids the culture was incubated at 26°C, 160 rpm for 7 h.

The cells were removed by centrifugation of the culture (15 min, 12000 g, 4°C). The supernatant containing the phagemid particles was sterile-filtered (0.45 µm), mixed with 1/4 volume (25 ml) of 20% w/v PEG 8000, 15% w/v NaCl and incubated at 4°C overnight. After centrifugation (20 min, 18000 g, 4°C), the precipitated phagemid particles were dissolved in a total of 4 ml of cold PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4). The solution was incubated on ice for 30 min and distributed into four 1.5 ml reaction vessels at equal

volumes. After removing undissolved components by centrifugation (5 min, 18500 g, 4°C), the supernatant was transferred in each case to a new reaction vessel.

The phagemid particles were again precipitated by mixing with 1/4 volume (in each case 0.25 ml per reaction vessel) of 20% w/v PEG 8000, 15% w/v NaCl and incubating on ice for 60 min. After centrifugation (20 min, 18500 g, 4°C), the supernatant was removed and the precipitated phagemid particles were each dissolved in 0.5 ml of PBS. After incubation on ice for 30 min, centrifugation (5 min, 18500 g, 4°C) was repeated to clarify the solution. The supernatant containing the phagemid particles (between $1 \cdot 10^{12}$ and $5 \cdot 10^{12}$ cfu/ml) was then used for affinity enrichment.

For affinity enrichment of the recombinant phagemids presenting the muteins of the bilin-binding protein Immuno-Sticks (NUNC) were used. These were coated overnight with 800 µl of a conjugate (100 µg/ml) of ribonuclease A (RNaseA) and digoxigenin in PBS.

The conjugate was prepared by adding 1.46 µmol (0.96 mg) of digoxigenin-3-O-methylcarbonyl-ε-amino-caproic acid N-hydroxysuccinimide ester (DIG-NHS, Boehringer Mannheim) in 25 µl of DMSO in µl steps and with constant mixing to 0.73 µmol (10 mg) of RNaseA (Fluka) in 1 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at room temperature (RT) for 1 h. Excess reagent was then removed from the RNaseA conjugate by means of a PD-10 gel filtration column (Pharmacia) according to the manufacturer's instructions.

Unoccupied binding sites on the Immuno-Stick surface were saturated by incubation with 1.2 ml of 2% w/v BSA in PBST (PBS with 0.1% v/v Tween 20) at RT for 2 h. After three short washes with in each case 1.2 ml of PBST, the Immuno-Stick was incubated in a mixture of

250 µl of phagemid solution and 500 µl of blocking buffer (2% w/v BSA in PBST) at RT for 1 h.

For removing unbound phagemids the solution was stripped off and the Immuno-Stick was washed eight times with in each case 950 µl of PBST for 2 min. Finally, adsorbed phagemids were competitively eluted during a 15 minute incubation of the Immuno-Stick with 950 µl of a 2 mM solution of digoxigenin in PBS (for this purpose, 0.742 mg of digoxigenin (Fluka) were dissolved in 19.2 µl of DMF and added to 930.8 µl of PBS).

The phagemids were propagated by heating 950 µl of solution of the elution fraction obtained (between 10^6 and 10^8 colony-forming units, depending on the selection cycle) briefly to 37°C, mixing the solution with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue (OD_{550} = 0.5) and incubated at 37°C, 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 µl of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

For repeated production and affinity enrichment of phagemid particles 50 ml of 2xYT/Amp medium prewarmed to 37°C were inoculated with 0.2 to 1 ml of said suspension so that the cell density was OD_{550} =0.08. This culture was incubated at 37°C, 160 rpm to a cell density of OD_{550} = 0.5, infected with 250 µl of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene), and the procedure was continued as already described above.

The phagemids obtained from the first affinity concentration were used to carry out a series of eight further enrichment cycles using Immuno-Sticks which had been freshly coated with the digoxigenin-RNaseA conjugate. The phagemids obtained after the last enrichment cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the colonies obtained was scraped off the agar plates using 2xYT/Amp medium and resuspended, as described above. This cell suspension was used to inoculate 50 ml of 2xYT/Amp medium, and the phasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions.

In order to be able to produce the muteins of the bilin-binding protein as a fusion protein with the Strep-tag II and the albumin-binding domain, the gene cassette between the two *Bst*XI cleavage sites was subcloned from vector pBBP20 into vector pBBP22. A relevant section of the pBBP22 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:9 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For this purpose, the DNA isolated from the mixture of the *E. coli* colonies was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis as described above. In the same manner, pBBP22 vector DNA was cut with *Bst*XI and the larger of the two fragments (3545 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 µl (30 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C overnight. 5 µl of this ligation

mixture were used to transform 200 µl of competent cells of *E. coli* strain TG1-F⁻ according to the $CaCl_2$ method (Sambrook et al., supra), and 2.2 ml of a cell suspension were obtained.

The transformants were then screened for production of muteins with binding activity for the digoxigenin group by means of a colony screening assay. For this purpose, a cut-to-fit hydrophilic PVDF membrane (Millipore, type GVWP, pore size 0.22 µm) marked at one position was placed on an LB/Amp agar plate. 150 µl of the cell suspension from the transformation mixture were plated out evenly on said membrane, and approx. 500 colonies were obtained. The plate was incubated in an incubator at 37°C for 7.5 h until the colonies were approx. 0.5 mm in diameter.

In the meantime, a hydrophobic membrane (Millipore, Immobilon P, pore size 0.45 µm) which had likewise been cut to fit was wetted with PBS according to the manufacturer's instructions. Said membrane was then gently agitated in a solution of 10 mg/ml of human serum albumin (HSA, Sigma) in PBS at RT for 4 h. Remaining binding sites on the membrane were saturated by incubation with 3% w/v BSA, 0.5% v/v Tween 20 in PBS at RT for 2 h. The membrane was washed with 20 ml of PBS for two times for 10 min and then gently agitated in 10 ml of LB/Amp medium to which 200 µg/l of anhydrotetracycline had been added for 10 min. Said membrane was then marked at one position and placed on a culture plate with LB/Amp agar which additionally contained 200 µg/l of anhydrotetracycline.

The previously obtained hydrophilic membrane on which colonies had grown was then placed onto the hydrophobic membrane such that the two markings coincided. The culturing plate with the two membranes was incubated at 22°C for 15 h. During this phase, the particular muteins were secreted by the colonies as fusion

proteins and immobilized on the lower membrane by means of complex formation between the albumin-binding domain and the HSA.

Subsequently, the upper membrane containing the colonies was transferred to a fresh LB/Amp agar plate and stored at 4°C. The hydrophobic membrane was removed, washed with 20 ml of PBST for three times 10 min and then incubated in 10 ml of a 10 µg/ml solution of a conjugate of BSA with digoxigenin in PBST for 1 h.

The conjugate of BSA (Sigma) and digoxigenin was prepared by adding a solution of 3.0 µmol (1.98 mg) of DIG-NHS in 25 µl of DMSO in µl steps and with constant mixing to 300 nmol (19.88 mg) of BSA (Sigma) in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h and excess reagent was removed from the BSA conjugate by means of a PD-10 gel filtration column according to the manufacturer's instructions.

In order to detect bound digoxigenin-BSA conjugate, the membrane was incubated, after washing twice in 20 ml of PBST, with 10 ml of anti-digoxigenin Fab-alkaline phosphatase conjugate (Boehringer Mannheim, diluted 1:1000 in PBST) for 1 h. The membrane was then washed twice with 20 ml PBST and twice with 20 ml of PBST for in each case 5 min and gently agitated in AP buffer (0.1 M Tris/HCl pH 8.8, 0.1 M NaCl, 5 mM $MgCl_2$) for 10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 µl of 5-bromo-4-chloro-3-indolyl phosphate, p-toluidinium salt (BCIP, Roth, 50 µg/ml in dimethylformamide) and 5 µl of Nitro Blue Tetrazolium (NBT, Sigma, 75 µg/ml in 70% v/v dimethylformamide) had been added, until at the positions of some of the colonies distinct color signals became visible. In this way, digoxigenin-binding activity of the bilin-binding

protein muteins which had been produced in the form of fusion proteins with Strep-tag and ABD by said colonies was detected.

Four colonies from the upper membrane, which caused a distinct color signal, were used for preparing cultures in LB/Amp medium of 4 ml in volume. Their plasmid DNA was isolated with the aid of the JETquick Plasmid Miniprep Spin kit (Genomed) according to the manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis. Sequence analysis was carried out with the aid of the T7 sequencing kit (Pharmacia) according to the manufacturer's instructions by using oligodeoxynucleotides SEQ ID NO:10 and SEQ ID NO:11. It was found in the process that all four plasmids studied carried the same nucleotide sequence. The corresponding gene product was denoted by DigA (SEQ ID NO:12). The DigA nucleotide sequence was translated into the amino acid sequence and is represented in the sequence listing.

Example 2: Partial random mutagenesis of the DigA mutein and selection of muteins with improved binding affinity for digoxigenin

In order to improve the affinity between the DigA mutein and digoxigenin, which was determined as 295 ± 36 nM according to Example 3, the 6 amino acid positions 28, 31 and 34-37 in DigA were selected for a more substantial partial random mutagenesis.

For mutating said positions the PCR was carried out using a degenerated oligodeoxynucleotide primer. The amplification reaction was carried out in a total volume of 100 µl, with 2 ng of the vector pBBP22 plasmid DNA coding for DigA (SEQ ID NO:12) being used as template. The reaction mixture contained 50 pmol of the two primers SEQ ID NO:13 and SEQ ID NO:7 and also

the other components according to the method described in Example 1. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 65°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min. The DNA fragment obtained was isolated by preparative agarose gel electrophoresis and then cut with *Bst*XI according to the manufacturer's instructions. The resulting DNA fragment of 335 bp in length was again purified by preparative agarose gel electrophoresis.

The pBBP24 vector DNA was cut with *Bst*XI accordingly and the 4028 bp fragment obtained was isolated. A relevant section of the pBBP24 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:14 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75. PBBP24 is virtually identical with pBBP20, wherein the BBP gene has been inactivated by means of appropriately introduced stop codons.

1.3 µg of the cleaved DNA fragment from the PCR and 16.0 µg of the pBBP24 fragment were incubated for ligation in the presence of 120 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 600 µl (50 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for 18 h. The DNA was then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for two weeks was followed by centrifugation (20 min, 16000 g, 4°C). The precipitate was washed in each case with 150 µl of ethanol (70% v/v, -20°C) and dried under vacuum. Finally, the DNA was taken up in 80 µl of TE/10.

E. coli XL1-Blue cells were transformed with the ligated DNA by electroporation according to the

procedure described in Example 1, with in each case 40 µl of cell suspension of electrocompetent cells being mixed with 5 µl of the DNA solution in 16 mixtures. After electroporation, the cells were immediately diluted in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

168 ml of 2xYT medium and 200 µl of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added to the combined suspensions. The total number of transformants obtained was estimated at $1.48 \cdot 10^9$ by plating out 100 µl of a $1:10^4$ dilution of the obtained cell suspension on agar plates. After incubation at 37°C and 160 rpm for 60 min, the transformants were infected with 4 ml of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene) and agitated at 37°C and 160 rpm for a further 30 min. Subsequently, 400 µl of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C and, after 10 min, anhydrotetracycline (100 µl of a 50 µg/ml stock solution in dimethylformamide, final concentration 25 µg/l) was added to induce gene expression. Finally, the phagemids were produced by incubating the culture at 26°C and 160 rpm for 7 h. The cells were removed and the phagemids purified by precipitation as described in Example 1.

Streptavidin-coated paramagnetic particles (Dynabeads M-280 Streptavidin, Dynal) were used together with a double conjugate of BSA with digoxigenin and biotin for affinity enrichment from the library of phagemids which presented the partially mutated DigA mutein.

A double conjugate of BSA with digoxigenin and biotin was prepared by adding 1.5 µmol (0.99 mg) of DIG-NHS in 12.5 µl of DMSO and 1.5 µmol (0.68 mg) of D-biotinoyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim) in 12.5 µl of DMSO in µl steps and with constant mixing to 300 nmol (19.88 mg)

of BSA in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h. Excess reagent was removed from the double conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

In order to enrich Digoxigenin-binding phagemids, 40 µl of a 0.5 µM solution of the double conjugate (33.5 µg/ml) in PBS were mixed with 260 µl of a solution of the freshly prepared phagemids (between $5 \cdot 10^{11}$ and $5 \cdot 10^{12}$ cfu/ml) and incubated at RT for 1 h so that the complex formation between the digoxigenin group and the muteins presented by the phagemids was able to occur. This was followed by adding 100 µl of a solution of 8% w/v BSA, 0.4% v/v Tween 20 in PBS.

Parallel thereto, 100 µl of the commercially available suspension of paramagnetic particles were washed with three times 100 µl of PBS. Here, the particles were kept suspended for 1 min by rotating the 1.5 ml Eppendorf vessel and then collected at the wall of the Eppendorf vessel with the aid of a magnet, and the supernatant was stripped off. In order to saturate unspecific binding sites, the paramagnetic particles were incubated with 100 µl of 2% w/v BSA in PBST at RT for 1 h. After removing the supernatant, the mixture of double conjugate and phagemids was added to the paramagnetic particles, and the particles were resuspended and incubated at RT for 10 min. Finally, free biotin-binding sites of Streptavidin were saturated by adding 10 µl of a 4 µM D-desthiobiotin (Sigma) solution in PBS to the mixture and incubating said mixture at RT for 5 min. This procedure also prevented the Strep-tag II as part of the fusion protein of the muteins and the phage coat protein pIII fragment from being able to form a complex with Streptavidin.

Unbound phagemids were removed by washing the paramagnetic particles eight times with 1 ml of fresh PBST with the addition of 1 mM D-desthiobiotin, the particles were collected with the aid of the magnet and the supernatant was stripped off. The bound phagemids were eluted by incubating the resuspended particles in 950 µl of 0.1 M glycine/HCl pH 2.2 for 15 minutes. After collecting the particles on the magnet, the supernatant was again stripped off and this was immediately followed by neutralizing the pH of said solution by addition of 140 µl of 0.5 M Tris.

The phagemids were propagated by mixing the elution fraction obtained, according to the procedure in Example 1, with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue (OD_{550} = 0.5) and incubating at 37°C, 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 µl of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

For repeated production and affinity enrichment of phagemid particles 50 ml of 2xYT/Amp medium prewarmed to 37°C was inoculated with 0.2 to 1 ml of said suspension so that the cell density was OD_{550} = 0.08. This culture was incubated at 37°C, 160 rpm to a cell density of OD_{550} = 0.5 and infected with 300 µl of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene). The affinity selection was then repeated using the paramagnetic particles and the digoxigenin/biotin double conjugate under the abovementioned conditions. A total of 4 selection cycles were carried out in this way.

The phagemids obtained after the last concentration cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the obtained colonies which had been scraped off the agar plates using 2xYT/Amp medium and had been resuspended, as described above, was used to inoculate 50 ml of 2xYT/Amp medium, and phasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions.

Subsequently, the gene cassette between the two *Bst*XI cleavage sites was subcloned, as in Example 1, from vector pBBP24 into vector pBBP22, and competent cells of *E. coli* strain TG1-F$^-$ were transformed according to the $CaCl_2$ method. Finally, the transformants were, again according to Example 1, screened for production of muteins with binding activity for the digoxigenin group by means of the colony screening assay.

Seven of the colonies showing a strong signal intensity in the colony screening assay were cultured. Their plasmid DNA was isolated by means of the plasmid miniprep spin kit (Genomed) according to the manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis as in Example 1. It was found in the process that all plasmids studied had different sequences. After translating the nucleotide sequences into amino acid sequences, six of the seven variants studied had an amber stop codon at amino acid position 28. However, this stop codon was at least partially suppressed when choosing suitable amber-suppressor strains such as, for example, *E. coli* XL1-Blue or TG1-F$^-$ and instead translated as glutamine. Thus a full-length functional protein was produced both during affinity enrichment and in the colony screening assay.

As the only mutein without an amber stop codon among the muteins found, the mutein with SEQ ID NO:15 was

particularly well suited for bacterial production. Consequently, this mutein, also denoted by DigA16, was characterized in more detail with regard to its ability to bind to the digoxigenin group.

Example 3: Production of the DigA and DigA16 muteins and determination of their affinity for digoxigenin and derivatives thereof by fluorescence titration

For preparative production of the bilin-binding protein muteins obtained from the previous Examples the coding gene section between the two *Bst*XI cleavage sites was sublcloned from the type pBBP22 vector into the expression plasmid pBBP21. The plasmid thus obtained coded for a fusion protein of the OmpA signal sequence, followed by the mutein and the Strep-tag II affinity tag.

A relevant section of the pBBP21 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:16 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with a hexanucleotide which was obtained by ligating a blunt strand end with a filled-up *Hind*III strand end, with the loss of the original *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For subcloning, the plasmid DNA coding for the relevant mutein was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis as described in Example 1. In the same manner, pBBP21 vector DNA was cut with *Bst*XI, and the larger of the two fragments (4132 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 µl (30 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$,

10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C for 16 h. 5 µl of the ligation mixture were then used to transform *E. coli* JM83 (Yanisch-Perron et al., Gene 33 (1985), 103-119) according to the $CaCl_2$ method, wherein 2.2 ml of a cell suspension were obtained. 100 µl of this suspension were plated out on an agar plate containing LB/Amp medium and incubated at 37°C for 14 h.

For protein production, one of the obtained single colonies was selected, a 50 ml preculture (LB/Amp medium) was inoculated with this colony and incubated at 30°C and 200 rpm overnight. 40 ml of the preculture were then transferred by inoculating 2 l of LB/Amp medium in a 5 l Erlenmeyer flask, followed by incubating the culture at 22°C and 200 rpm. At a cell density of OD_{550} = 0.5, gene expression was induced by adding 200 µg/l anhydrotetracycline (200 µl of a 2 mg/ml stock solution in DMF), followed by agitating at 22°C, 200 rpm for a further 3 h.

The cells were removed by centrifugation (15 min, 4420 g, 4°C) and, after removing the supernatant, resuspended in 20 ml of periplasm lysis buffer (100 mM Tris/HCl pH 8.0, 500 mM sucrose, 1 mM EDTA) with cooling on ice. After incubation on ice for 30 min, the spheroplasts were removed in two successive centrifugation steps (15 min, 4420 g, 4°C and 15 min, 30 000 g, 4°C). The periplasmic protein extract obtained in this way was dialyzed against SA buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), sterile-filtered and used for chromatographic purification.

Purification was carried out by means of the Strep-tag II affinity tag (Schmidt and Skerra, Protein Eng. 6 (1993), 109-122) fused to the C-terminus of the muteins. In the present case, Streptavidinmutein "1" was used (Voss and Skerra, Protein Eng. 10 (1997), 975-

982), which was coupled to activated Sepharose (with 5 mg/ml immobilized Streptavidin with respect to the bed volume of the matrix).

A chromatography column packed with 2 ml of said material was equilibrated at 4°C and a flow rate of 20 ml/h with 10 ml of SA buffer. The chromatography was monitored by measuring absorption of the eluate at 280 nm in a flow-through photometer. Application of the periplasmic protein extract was followed by washing with SA buffer until the base line was reached. Bound mutein was then eluted with 10 ml of a solution of 2.5 mM D-desthiobiotin (Sigma) in SA buffer. The fractions containing the purified mutein were checked by means of SDS polyacrylamide gel electrophoresis (Fling and Gregerson, Anal. Biochem. 155 (1986), 83-88) and combined. The protein yields were between 200 µg and 800 µg per 2 l culture.

The ligand binding properties of muteins DigA, DigA16 and also of the recombinant bilin-binding protein (SEQ ID NO:16) were determined by means of the method of fluorescence titration. In this case, the decrease in intrinsic tyrosine and/or tryptophan fluorescence of the protein forming a complex with the ligand was measured. The measurements were carried out in a fluorimeter, type LS 50 B (Perkin Elmer) at an excitation wavelength of 295 nm (slit width 4 nm) and an emission wavelength of 345 nm (slit width 6 nm). The ligands used were digoxigenin (Fluka), digoxin (Fluka), digitoxigenin (Fluka), digitoxin (Fluka), testosterone (Sigma), ouabain (Fluka), and 4-aminofluorescein (Fluka). The ligands showed no significant intrinsic fluorescence or absorption at the stated wavelength.

The buffer system used was PBS with the addition of 1 mM EDTA. The solution of the relevant purified mutein was dialyzed four times against this buffer and adjusted to a concentration of 1 µM by dilution. All

solutions used were sterile-filtered (Filtropur S 0.45 µm, Sarstedt). The concentration was determined by means of absorption at 280 nm using calculated extinction coefficients of 53580 M^{-1} cm^{-1} for DigA and DigA16 (Wisconsin Software Package, Genetics Computer Group). For Bbp, the calculated extinction coefficient of 54150 M^{-1} cm^{-1}, corrected in the presence of guanidinium chloride according to Gill and von Hippel (Anal. Biochem. 182 (1989), 319-326) was used.

For the measurement, 2 ml of the mutein solution were introduced into a quartz cuvette equipped with a magnetic stirrer bar and thermally equilibrated at 25°C in the sample holder of the photometer. Then a total of 40 µl of a 100 µM to 500 µM solution of the ligand in the same buffer were pipetted in steps of from 1 µl to 4 µl. The dilution of the introduced protein solution by altogether no more than 2%, which took place in the process, was not taken into account in the subsequent evaluation of the data. After each titration step, the equilibrium was allowed to reach by incubating with stirring for 1 min, and the fluorescence signal was measured as average over 10 s. After subtracting the fluorescence value of the buffer, the signals were normalized to an initial value of 100%.

The thus obtained data of a titration series were fitted by nonlinear regression using the computer program Kaleidagraph (Abelbeck Software) according to the following equation

$$F = ([P]_t - [L]_t - K_d)\frac{f_P}{2} + ([P]_t + [L]_t + K_d)\frac{f_{PL}}{2} + (f_P - f_{PL})\sqrt{\frac{([P]_t + [L]_t + K_d)^2}{4} - [P]_t[L]_t}$$

Here, F means the normalized fluorescence intensity and $[L]_t$ the total ligand concentration in the particular titration step. $[P]_t$ as mutein concentration, f_{PL} as fluorescence coefficient of the mutein-ligand complex

and K_d as the thermodynamic dissociation constant of said complex were fitted as free parameters to the normalized data.

Figure 1 represents graphically the results of the fluorescence titrations of the DigA16 mutein with the ligands digoxigenin, digitoxigenin and ouabain. It turns out that digitoxigenin is bound even tighter than digoxigenin, while no binding is observed for ouabain.

The values resulting from the fluorescence titrations for the dissociation constants of the complexes of the bilin-binding protein muteins and the various ligands are summarized in the following table:

Bbp variant	Ligand	K_d [nM]
Bbp:	digoxigenin	-*
DigA:	digoxigenin	295 ± 37
	digoxin	200 ± 34
DigA16:	digoxigenin	30.2 ± 3.6
	digoxin	31.1 ± 3.2
	digitoxigenin	2.8 ± 2.7
	digitoxin	2.7 ± 2.0
	ouabain	-*
	testosterone	-*
	4-aminofluroescein	-*

* no detectable binding activity

Example 4: Preparation of fusion proteins of the DigA16 mutein and bacterial alkaline phosphatase and use for detecting digoxigenin groups in an ELISA and in a Western blot

In order to produce two different fusion proteins of the DigA16 mutein and bacterial alkaline phosphatase (PhoA) with different arrangement of the partners within the polypeptide chain, the two expression plasmids pBBP27 and pBBP29 were constructed by using

the molecular-biological methods familiar to the skilled worker.

pBBP27 codes for a fusion protein of PhoA including the signal sequence thereof, a short peptide linker having the amino acid sequence Pro-Pro-Ser-Ala, the sequence corresponding to the mature DigA16 mutein and the Strep-tag-II. A relevant section of the pBBP27 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:17 in the sequence listing. The section begins with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pBBP21.

pBBP29 codes for a fusion protein of DigA16 with preceding OmpA signal sequence, followed by the peptide sequence for Strep-tag II, a sequence of 5 glycine residues and the mature PhoA sequence without the N-terminal amino acid arginine. A relevant section of the pBBP29 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:18 in the sequence listing. The section begins with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pBBP21.

Both plasmids additionally code for the bacterial protein disulfide isomerase DsbC on a separate cistron located in 3' direction. The plasmids are shown diagrammatically in Figure 2.

The fusion proteins encoded by plasmids pBBP27 and pBBP29 were produced analogously to the method for preparing the simple muteins, described in Example 3. In order to avoid complexing the metal ions from the active center of PhoA, lysis of the bacterial periplasm was carried out using EDTA-free lysis buffer. Polymyxin B sulfate (2 mg/ml, Sigma) was added to the

buffer as an agent destabilizing the outer cell membrane. All other buffers used for purification were likewise EDTA-free.

The fusion proteins purified by affinity chromatography by means of the Strep-tag II were dialyzed against PBS buffer overnight. The fusion protein yields were between 100 and 200 µg per 2 l of culture medium. The purity of the fusion proteins obtained was checked by SDS polyacrylamide gel electrophoresis, according to Example 3, and determined to be 90-95%. Subsequently, the fusion proteins were used for directly detecting conjugates of the digoxigenin group with various proteins both in a sandwich ELISA and in a Western blot.

While the conjugates used of digoxigenin with RNaseA and BSA were prepared according to Example 1, a conjugate of digoxigenin with ovalbumin (Sigma) was prepared by adding 1.5 µmol (0.99 mg) DIG-NHS in 25 µl of DMSO in µl steps and with constant mixing to 300 nmol (13.5 mg) of ovalbumin in 1.9 ml of 5% sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h. Excess reagent was removed from the ovalbumin conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

For detecting digoxigenin groups in a sandwich ELISA, the wells in in each case two rows of a microtiter plate (ELISA strips, 2×8 well with high binding capacity, F-type, Greiner) were filled in each case with 100 µl of a 100 µg/ml solution of the BSA-digoxigenin conjugate or the ovalbumin-digoxigenin conjugate in PBS and incubated at RT overnight. As a control, the wells of a fifth vertical row of the microtiter plate were filled with 100 µl of a 100 µg/ml solution of nonconjugated BSA (Sigma) in PBS and likewise incubated at RT overnight. After removing the solution, unoccupied binding sites were saturated with

200 µl of a solution of 2% w/v BSA in PBST for 2 h. After washing three times with PBST, 50 µl of a 1 µM solution of the purified fusion protein were in each case introduced into the first well of a row, and the Tween concentration was adjusted to 0.1% v/v by adding 1 µl of a solution of 5% v/v Tween. The subsequent wells in each row were initially charged with 50 µl of PBST. Then, 50 µl of the purified fusion protein were pipetted in each case into the second well, mixed and, starting therefrom, 1:2 dilutions were prepared stepwise in the other wells of the vertical row. After incubation at RT for 1 h, the wells were washed twice with PBST and twice with PBS. The fusion proteins bound to the digoxigenin groups were finally detected by means of alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate. For this purpose, 100 µl of a solution of 0.5 mg/ml p-nitrophenyl phosphate (Amresco) in AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl pH 8.8) were introduced into the wells and product formation was monitored by measuring absorption at 405 nm in a SpectraMax 250 photometer (Molecular Devices).

Figure 3 shows the result of this measurement. According to this, the digoxigenin group is recognized both as conjugate with BSA and as conjugate with ovalbumin, leading to the conclusion that binding by the DigA16 mutein is context-independent. Furthermore, both fusion proteins are active both with regard to the binding function for the digoxigenin group and enzymatically and produce, despite their different structure, almost identical signals.

In order to use the fusion proteins encoded by vectors pBBP27 and pBBP29 in a Western blot, 5 µl of a protein mixture in PBS, whose concentration of digoxigenin-BSA conjugate, digoxigenin-ovalbumin conjugate and digoxigenin-RNaseA conjugate was simultaneously in each case 100 µg/ml, as well as 5 µl of a protein mixture in

PBS, whose concentration of underivatized BSA, ovalbumin and RNaseA likewise was simultaneously in each case 100 µg/ml, were first separated by SDS polyacrylamide gel electrophoresis. The protein mixture was then transferred to nitrocellulose by electrotransfer (Blake et al., Anal. Biochem. 136 (1984), 175-179). The membrane was then washed in 10 ml of PBST for three times 5 min and incubated with 10 ml of a 0.5 µM solution of in each case one of the two fusion proteins for 1 h. The membrane was then washed in 10 ml PBST for two times 5 min and in 10 ml of PBS for two times 5 min and finally gently agitated in 10 ml of AP buffer for 10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 µl BCIP (50 µg/ml in dimethylformamide) and 5 µl NBT (75 µg/ml in 70% v/v dimethylformamide) had been added, and bound fusion protein was detected in this way.

Figure 4 shows the result of this detection method. It turns out again that binding of the digoxigenin group by the two fusion proteins is independent of the carrier protein and that both fusion proteins achieve comparable signal intensities. The same carrier proteins cause no signal whatsoever if they are not conjugated with the digoxigenin group.